AD_____

Award Number: DAMD17-03-1-0249

TITLE: Cellular Retinoic Acid Binding Protein and Breast Cancer

PRINCIPAL INVESTIGATOR: Leslie J. Willmert, Ph.D.

CONTRACTING ORGANIZATION: Cornell University
Ithaca, NY 14853

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050916 170

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual Summary (14 Apr 2004 - 13 Apr 2005)

4. TITLE AND SUBTITLE
Cellular Retinoic Acid Binding Protein and Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0249

6. AUTHOR(S)
Leslie J. Willmert, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cornell University
Ithaca, NY 14853

E-Mail: Ljw23@cornell.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In attempts to understand how the signaling by retinoic acid (the active vitamin A metabolite) is regulated we have been studying the retinoic acid binding protein called CRABP-II. These studies revealed that CRABP-II acts to enhance the transcriptional activities of RA and that it does so by directly delivering the hormone to its cognate transcription factor, RAR. Consequently CRABP-II dramatically sensitized cultured mammary carcinoma cells to RA-induced growth inhibition. Similarly, over-expression of CRABP-II inhibited mammary tumor growth in two different mouse models of cancer. CRABP-II may be a novel target for therapeutic and preventive strategies for retinoid-treatment of breast cancer. This project aims to delineate the mechanism by which CRABP-II modulates RA activity, especially as related to its ability to enhance the anti-proliferative action of the ligand.

14. SUBJECT TERMS
Transcription regulation, RAR, retinoic acid, CRABP, apoptosis

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Retinoic acid (RA), the active metabolite of vitamin A, plays critical roles in embryonic development as well as growth and differentiation in adult mammals. Retinoic acid is currently used or is in clinical trials for treatment of a variety of different cancers, including breast cancer (*1*). Although retinoids are efficacious, pharmacological doses often result in toxicity (*2*). The anticarcinogenic activities of RA are mediated by the ligand-inducible transcription factors termed retinoic acid receptors (RARs) (*3*). Like other type-II nuclear receptors, RARs function as heterodimers with the retinoid X receptor (RXR). These heterodimers associate with specific DNA sequences (RAR response elements, RARE) comprised of two direct repeats of the consensus sequence PuG(G/T)TCA, separated by either 2 (DR-2) or 5 (DR-5) basepairs (*3, 4*). RXR-RAR heterodimers thus bind in regulatory regions of their target genes and enhance transcriptional rates upon binding of RA (*5*). In cells, RA also associates with cellular retinoic acid binding proteins (CRABP-I and CRABP-II). We recently showed that CRABP-II carries RA from the cytoplasm to the nucleus where it channels the ligand directly to RARα via a transient protein-protein interaction (*6, 7*). This "ligand channeling" sensitizes cells to transcriptional activation by RAR (*6*). Indeed, over-expression of the binding protein dramatically lowered the effective RA concentration necessary to induce growth inhibition in mammary carcinoma cells (*7*). Similarly, over-expressing CRABP-II inhibited mammary tumor growth in two different mouse models of cancer. The goal of this project is to elucidate the mechanisms by which CRABP-II directs RA signaling to enhance anti-proliferative responses in mammary carcinoma cells.

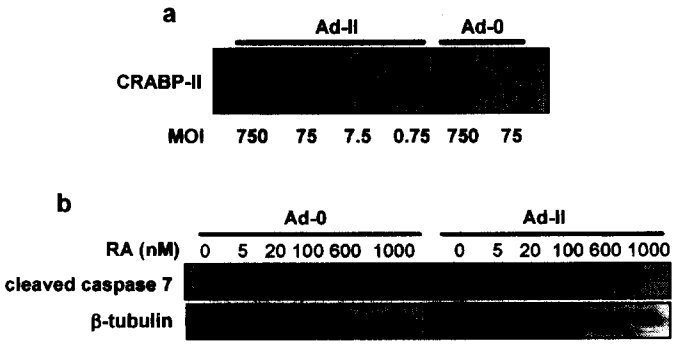
Body

To determine the mechanism by which CRABP-II inhibits growth of mammary carcinoma cells.

Our group has previously shown that over-expression of CRABP-II enhances the ability of RA to inhibit the growth of the mammary carcinoma cell line MCF-7 (*7*). The goal of this project is to determine the mechanisms by which RA inhibits the growth of these cells. We tested two signaling pathways that may respond to RA through RAR-mediated transcriptional regulation to induce growth arrest: (1) Apoptosis- RA may induce programmed cell death, characterized by cleavage of cytosolic cysteine proteases, caspases, and by DNA fragmentation. (2) Cell cycle arrest- RA could induce growth arrest by blocking the cell cycle at a particular stage.

Studies carried out last year indicate that RA causes MCF-7 cells to undergo apoptosis after five days of treatment. This was indicated by flow cytometry studies which revealed significant RA-induced DNA fragmentation. Apoptotic responses are coordinated by a tightly regulated group of cysteine proteases named caspases. In a normal healthy cell, these proteins are found in an inactive state. Upon stimulation of apoptosis, procaspases are cleaved to produce active proteases which then cleave cellular targets to propagate the apoptotic signal and induce death. Therefore, apoptotic responses can be detected by monitoring the appearance of cleaved caspases. Treatment of MCF-7 cells with RA for 5 days caused a dose dependent activation of caspase 9 and cells that ectopically over-expressed CRABP-II displayed enhanced caspase 9 cleavage. Therefore, we concluded at the end of last year that RA-induced apoptosis in MCF-7 cells is a caspase 9 mediated event and that over-expression of CRABP-II enhances the caspase response. We have since discovered that RA also induces cleavage of caspase 7 and that CRABP-II dramatically enhances this activity (Figure 1). CRABP-II was overexpressed by using an adenovirus

Figure 1 CRABP-II Enhances RA-Induced Caspase 7 Cleavage



encoding the gene's cDNA (Figure 1a). Caspase 7 cleavage was slightly induced upon RA treatment (Figure 1b left panel). Upon over-expression of CRABP-II, caspase 7 cleavage was greatly enhanced (Figure 1b right panel). Therefore, RA-induced apoptosis is associated with several caspase activation events including caspase 9 and caspase 7, and CRABP-II cooperates with RA in mediating the response.

Effect of RA and CRABP-II on gene expression profiles in MCF-7 cells.

To identify the genes involved in these responses, we carried out expression array studies using Affymetrix cDNA hybridization chips to identify genes that are up-regulated in response to RA. As reported in last year's annual report, the expression of many genes involved in apoptotic responses were induced. Specifically, caspase 7 and caspase 9 genes were both induced in response to RA. These observations suggest a mechanism by which RA triggers the apoptotic response in MCF-7 cells. In the past year we have examined the mechanism by which these genes are induced by RA. Regulation of expression of these genes by RA may be exerted directly, i.e. mediated by an RAR response element (RARE). Alternatively, responses may reflect secondary events involving RAR control of immediate target genes, which, in turn, are involved in secondary events leading to the observed modulation. Hence, an important question that arises is which of the RA-controlled pro-apoptotic genes in MCF-7 cells comprise direct targets for RAR. This question is particularly pertinent considering the present paucity of information on the mechanisms by which RA exerts its anticarcinogenic activities. To begin to address this issue, we examined whether either caspase 7 or caspase 9 are under direct RAR control. To validate the array data, quantitative real-time PCR (Q-PCR) was carried out. MCF-7 cells were treated for 4 hr with RA (50 nM) or vehicle and Q-PCR was used to compare the expression levels of mRNA for caspase 9 and caspase 7 in RA-treated *vs.* untreated cells (Fig. 2). In good agreement with the Affymetrix array data, the expression levels of mRNA for both caspase 9 and caspase 7 increased by about 60% in response to RA treatment. To determine whether these genes are direct targets for transcriptional regulation by RA, the effect of the protein synthesis inhibitor cycloheximide on their induction by RA was studied. Cycloheximide treatment will abolish secondary events that require *de novo* protein synthesis, but will not affect direct transcriptional responses. The analyses showed that inhibition of protein synthesis completely abolished the RA-response of caspase 7 expression (Fig. 2a). In contrast, caspase 9 mRNA was up-regulated by RA regardless of the presence of cycloheximide (Fig. 2b). Hence, while the effect of RA on caspase 7 expression is a secondary response, *caspase 9* is likely to be a direct target for RAR signaling.

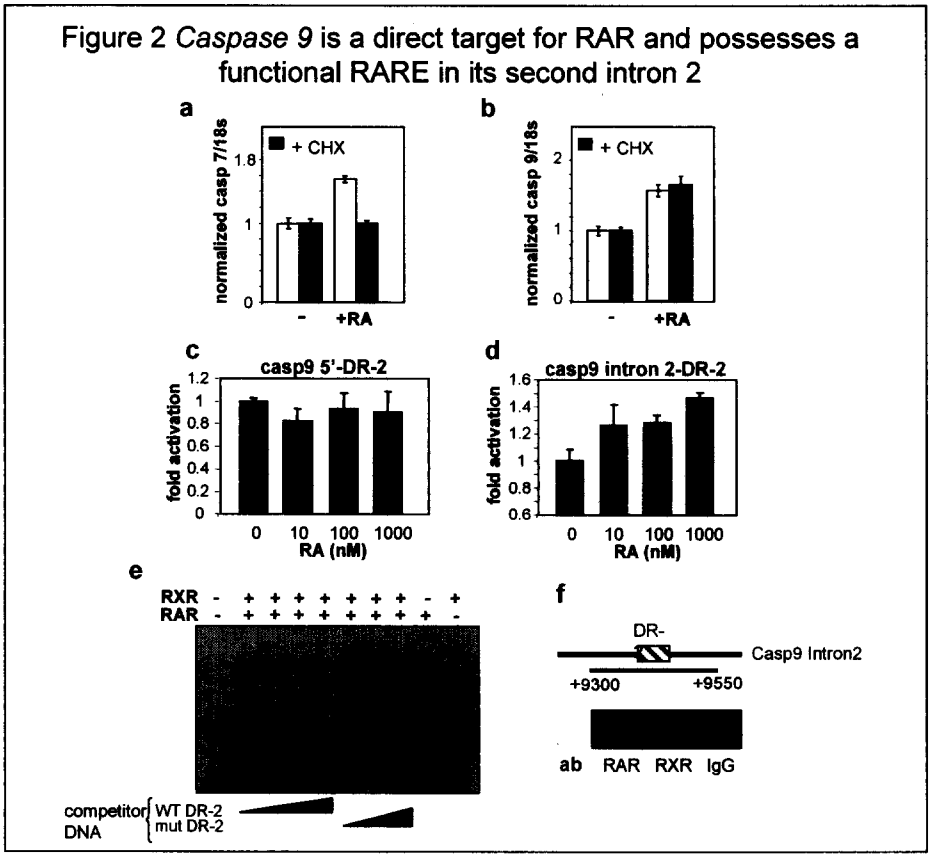


Figure 2 *Caspase 9* is a direct target for RAR and possesses a functional RARE in its second intron 2

Consensus RAREs are comprised of two direct repeats of the sequence PuG(G/T)TCA spaced by either 2 (DR-2) or 5 (DR-5) basepairs. We used two programs, TransFac (www.gene-regulation.com, (*8*)) and TESS (www.cbil.upenn.edu/tess, (*9*)), to screen the human *caspase 9* gene and adjacent regulatory sequences for potential RAREs. Within a stretch of eight kilobases upstream of the *caspase 9* start site (1p36.3), a potential RARE comprised of the non-canonical DR-2 sequence AGGTCAgcAGTTCG was found at position -1690. This element along with 38 basepairs of flanking

sequences on both sides was cloned into a luciferase reporter vector and its functionality was examined by transactivation assays carried out in MCF-7 cells. The expression of the reporter did not respond to RA (Fig 2c), suggesting that the element does not function as an RARE. An additional potential RARE, comprised of the consensus DR-2 sequence AGGTCAggAGTTCA was found in the second intron of the *caspase 9* gene, 9461 bp downstream of the start site. This RARE and 45 basepairs of flanking sequences on each side were cloned into a luciferase reporter which was used in transactivation assays. The data (Fig. 2d) demonstrated a dose-responsive activation of reporter expression by RA, suggesting that the element indeed comprises a functional RARE. To verify that the element can bind RAR-RXR heterodimers, electrophoretic mobility-shift assays (EMSA) were carried out. RARα and RXRα lacking their amino terminal A/B domains (RARαΔAB and RXRαΔAB) were expressed in *E. coli*, purified, and examined for binding to a 90 basepair oligonucleotide harboring the putative response element and its flanking sequences. The data (Fig. 2e) demonstrated that RAR-RXR heterodimers tightly and specifically associate with the DR-2 element of the second intron of *caspase 9*. Finally, to examine whether the element is occupied by RXR-RAR heterodimers in a living cell, chromatin immunoprecipitation (ChIP) assays were carried out. Proteins were crosslinked to chromatin in MCF-7 cells and immunoprecipitated using antibodies for RAR or RXR, or non-specific IgG. Precipitates were sonicated, the crosslink reversed, DNA isolated, and a 250 bp region surrounding the DR-2 in the second intron of *caspase 9* amplified by PCR. The data (Fig. 2f) showed that antibodies against either RAR or RXR specifically precipitated the intron DR-2 sequence, demonstrating that the element is occupied by the heterodimers in cells. Taken together, the observations establish that caspase 9 is a direct target for RAR signaling, and that the RARE responsible for this response is likely to be a DR-2 element located in the second intron of the gene.

Understand the underlying basis for RA resistance in mammary carcinoma cells

A frequent complication in utilizing RA for cancer therapy is the development of RA-resistance in tumors. Understanding the mechanisms that underlie RA-resistance in cancer cells are thus of significant clinical importance. We have observed that mammary carcinoma cells that over-express CRABP-II become more sensitive to RA-induced growth inhibition. Conversely, we find that cells that do not express CRABP-II are resistant to this anti-proliferative effect. Therefore, we examined the effect of RA on the expression of CRABP-II. As reported last year, RA treatment of MCF-7 cells resulted in a decrease in CRABP-II protein as well as a decrease in CRABP-II mRNA levels. We have since discovered that RA causes CRABP-II protein and mRNA levels to decrease in other cells including the human keratinocyte cell line HaCat (Figure 3). This down-regulation of CRABP-II in response to RA suggests a novel mechanism of negative feedback regulation of RA signaling. The decrease in CRABP-II mRNA levels could arise by two different mechanisms: (1) RA could affect the rate of transcription of the CRABP-II gene (2) RA could modulate the stability of the

Figure 3 RA causes down-regulation of CRABP-II in HaCat cells

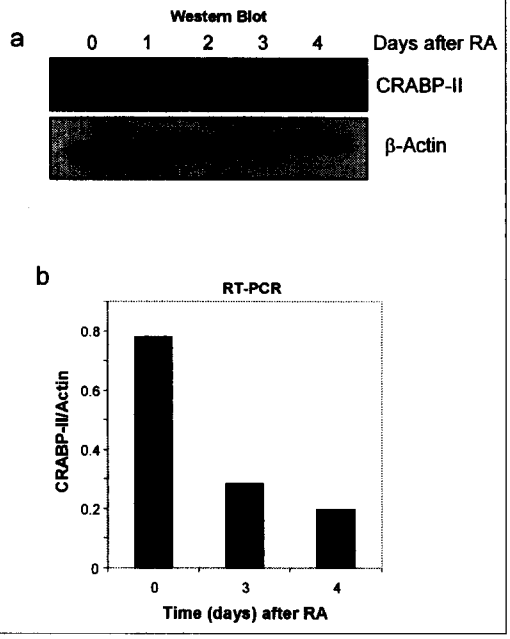
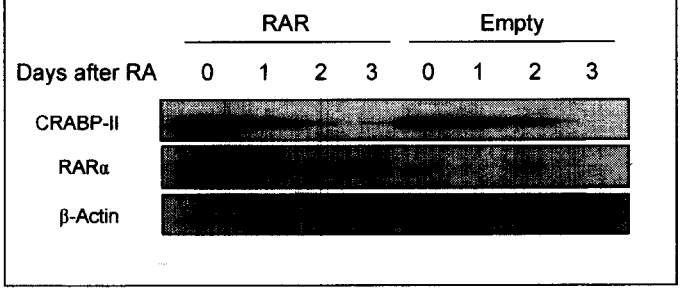
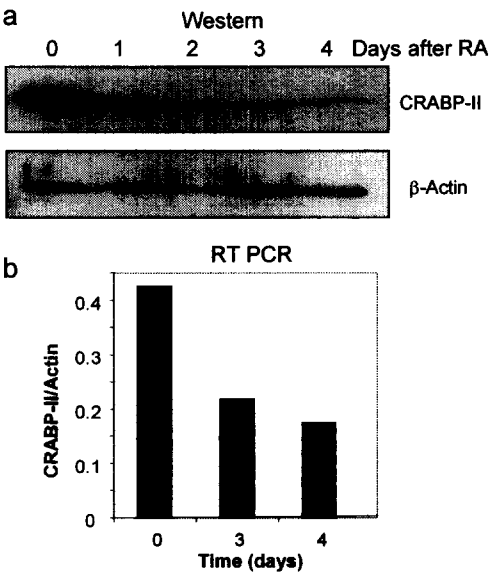


Figure 4 Exogenous RAR does not rescue CRABP-II expression



CRABP-II mRNA. It has been previously shown that RAR itself is degraded in response to RA treatment via the ubiquitin-proteosome pathway. CRABP-II is regarded as a RA-induced gene controlled by an RARE in its promoter region. Therefore, we hypothesized that RA could decrease CRABP-II mRNA levels by causing the degradation of the transcription factor (RAR) that is needed for its expression. To test this hypothesis, we transfected exogenous RAR into MCF-7 cells and monitored the CRABP-II protein and mRNA levels to see if replenishing RAR levels would rescue CRABP-II expression. Even in cells that over-express RAR, CRABP-II protein and mRNA levels continued to decrease in response to RA (Figure 4). Therefore, replenishing RAR did not rescue CRABP-II expression.

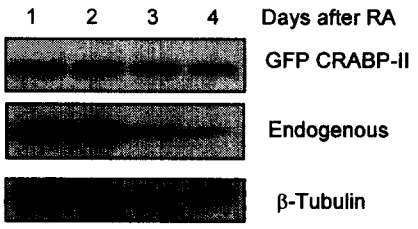
Figure 5 CRABP-II down-regulation by RA in SC115.C1 cells



To test directly if CRABP-II mRNA levels decrease because of a decline in the transcriptional rate of the endogenous gene, we utilized a cell line that does not express endogenous CRABP-II, but that is stably transfected with the CRABP-II cDNA. In this cell line (SC115.C1), the expression of CRABP-II is not under the control of its native promoter. If RA affects the rate of transcription of the CRABP-II gene, we would expect the transcript level in this cell line to be unresponsive to RA treatment. The date showed that CRABP-II protein and mRNA levels decrease in response to RA in the SC115.C1 cell line (Figure 5). This result suggests that CRABP-II mRNA levels are not affected by RA at the transcriptional level and points to mRNA stability as the target of RA actions on the CRABP-II transcript.

Message RNA stabilities are often regulated by RNA-binding proteins, many of which bind to the 3' untranslated region (UTR). One such protein, nucleolin, has been shown to stabilize the BCL-2 transcript by binding to an AU-rich region in the transcripts 3'-UTR(*10*). It has also been shown that upon treatment with RA, nucleolin levels decrease resulting in destabilization of the BCL-2 transcript and reduction of its expression level (*11*). The CRABP-II transcript has a similar AU-rich region in its 3' UTR. To test whether the UTRs of the CRABP-II transcript are important for its stability, we transfected MCF-7 cells with a GFP-tagged CRABP-II construct that does not contain the gene's untranslated regions. Upon treatment with RA, the endogenous levels of CRABP-II decreased as have been observed before; however, the GFP-CRABP-II construct was unresponsive to RA treatment (Figure 6). These observations indicate that the UTRs of CRABP-II could be responsible for the stability of the transcript. RA-induced down-regulation of CRABP-II expression may thus comprise an important feature through which carcinoma cells are rendered RA-resistant. On going studies aim to test the effects of CRABP-II UTRs in its mRNA stability.

Figure 6 GFP-CRABP-II is not down-regulated by RA



Key Research Accomplishments

- Caspase 7 activation is induced upon RA-induced apoptosis in MCF-7 cells.
- Caspase 9 is a direct target for RA signaling and CRABP-II enhances its expression. This is the first demonstration of CRABP-II facilitating RAR-mediated transcription of an endogenous gene.
- Caspase 9 contains a functional RARE in its second intron.
- Over-expression of CRABP-II in MCF-7 cells enhances the capase 7-mediated apoptotic response to RA.
- RA induces the down-regulation of CRABP-II expression in a variety of cells.
- RA-mediated down-regulation of CRABP-II is not exerted through effects on the transcription of the gene.
- RA-mediated down-regulation of CRABP-II may be due to decreasing its mRNA stability.
- RA-resistance of carcinoma cells may stem from RA-induced down-regulation of CRABP-II.

Reportable Outcomes:

Willmert and Noy, 2005, Suppression of mammary carcinoma growth by retinoic acid: pro-apoptotic genes are targets for RAR and CRABP-II signaling, Cancer Research, *in review*

Conclusions:

RA is currently used or is in clinical trials for therapy of a variety of cancers, however, at pharmacological doses it is often toxic and tumors often develop resistance to the treatment. Our lab is investigating approaches that will allow for sensitization of cancer cells to RA chemotherapy in order to increase the therapeutic efficacy of this compound. We have found that the RA binding protein CRABP-II functions to inhibit mammary carcinoma cell proliferation in culture as well as tumor progression *in vivo*. Therefore, CRABP-II may be a novel target for therapeutic and preventive strategies for treatment of breast cancer. The mechanisms by which this binding protein acts to modulate RA signaling and enhance the anti-proliferative activities of this compound in breast cancer are being studied. CRABP-II enhances RA-induced apoptosis in MCF-7 cell via caspase-mediated events. One of these caspase, caspase 9, was shown to be a novel direct target for RA signaling. Studies carried out during this funding period have begun to elucidate the mechanism by which RA down-regulates CRABP-II expression in carcinoma cells. This effect is not due to decreased transcriptional activation of the gene, but rather, RA affects the stability of the CRABP-II mRNA. This activity may be an important factor in the development of RA-resistance in carcinomas.

References:

1. L. M. Yang, U. C. Tin, K. Wu, P. Brown, *J Mammary Gland Biol Neoplasia* **4**, 377-88 (Oct, 1999).
2. W. K. Hong, L. M. Itri, in *The Retinoids: Biology Chemistry and Medicine* M. B. Sporn, A. B. Roberts, D. S. Goodman, Eds. (Raven Press, New York, 1994) pp. 597-630.
3. P. Chambon, *Faseb J* **10**, 940-54 (Jul, 1996).
4. H. de The, M. M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, A. Dejean, *Nature* **343**, 177-80 (Jan 11, 1990).
5. D. J. Mangelsdorf *et al.*, *Cell* **83**, 835-9 (Dec 15, 1995).
6. D. Dong, S. E. Ruuska, D. J. Levinthal, N. Noy, *J Biol Chem* **274**, 23695-8 (Aug 20, 1999).
7. A. S. Budhu, N. Noy, *Mol Cell Biol* **22**, 2632-41 (Apr, 2002).
8. E. Wingender *et al.*, *Nucleic Acids Res* **29**, 281-3 (Jan 1, 2001).
9. M. Podvinec, M. R. Kaufmann, C. Handschin, U. A. Meyer, *Mol Endocrinol* **16**, 1269-79 (Jun, 2002).
10. T. K. Sengupta, S. Bandyopadhyay, D. J. Fernandes, E. K. Spicer, *J Biol Chem* **279**, 10855-63 (Mar 19, 2004).
11. Y. Otake, T. K. Sengupta, S. Bandyopadhyay, E. K. Spicer, D. J. Fernandes, *Mol Pharmacol* **67**, 319-26 (Jan, 2005).